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Fabrication of Novel Porous Chitosan Matrices as Scaffolds for Bone Tissue Engineering

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ABSTRACT

Three dimensional (3-D) scaffolds with appropriate mechanical properties play a significant role in scaffold-based tissue engineering. Chitosan, a natural polymer obtained from chitin, which forms a major component of crustacean exoskeleton, is a potential candidate for bone tissue engineering due to its excellent osteocompatibility and biodegradability. The aim of the present study is to develop 3-D porous chitosan scaffolds with mechanical properties in the range of trabecular bone as scaffolds for bone tissue engineering. Three dimensional scaffolds were prepared by sintering chitosan microspheres. Chitosan microspheres were prepared by ionotropic gelation of chitosan solution using sodium tripolyphosphate. It has been found that the microsphere size increased significantly with the increase of the concentration of chitosan solution. The microspheres were then sintered together using the synergetic effect of solvent and temperature. The compressive moduli of the 3-D sintered matrices were found to be in the mid range of trabecular bone. The osteocompatibility and osteoconductivity of the 3-D matrices were demonstrated by adhesion and proliferation of MC3T3-E1 osteoblast like cells on the matrices after 14 days in culture.

INTRODUCTION

Over 800,000 bone grafting procedures are performed each year in the United States [1]. Current healing therapies using autografts or allografts, although fairly successful, have various limitations associated with them such as the insufficient supply of the donor tissue, donor site morbidity in the case of autografts and risk of rejection and disease transmission in the case of

allografts. Recently, tissue engineering has then developed as an alternative therapeutic approach for skeletal regeneration. Tissue engineering has been defined as the application of biological, chemical, and engineering principles toward the repair, restoration, or regeneration of living tissue by using biomaterials, cells, and factors alone or in combination [2]. In scaffold based tissue engineering a porous three dimensional matrix developed from natural or synthetic materials provide support and guidance for tissue regeneration by mimicking structure and functions of the natural extracellular matrix.

Natural biopolymer chitosan is the N-deacetylated derivatives of chitin, the second most abundant natural polymer originating from crustacean shells. Chitosan has received much research interests recently due to its biocompatibility [3-4], biodegradability [5], antimicrobial and antifungal activities [6-7]. In addition, the possibility of chemically modifying chitosan at its amino or hydroxyl groups offers great flexibility to this biopolymer, making it an excellent candidate for a wide range of biomedical applications.

The excellent osteocompatibility of chitosan has been demonstrated earlier [8-9]. Therefore various attempts are currently underway to develop porous three dimensional matrices from chitosan as potential candidates for tissue engineering. A number of studies have demonstrated the possibility of fabricating porous chitosan matrices using freeze drying method [10-11]. However, the freeze drying process results in the formation of high porous matrices with low mechanical properties, which limits their use in load-bearing bone regeneration applications. The objective of the present study was to develop porous chitosan matrices with interconnected pore structure and having mechanical properties in the mid range of trabecular bone (10-2000 MPa) [12] as novel scaffolds for bone tissue engineering.

MATERIALS AND METHODS

Preparation of chitosan microspheres

Chitosan microspheres were prepared by the ionotropic gelation of chitosan solution using tripolyphosphate ions as the crosslinking agent [13]. Briefly, chitosan (Sigma, St. Louis, MO) solutions in 1% (v/v) acetic acid with different concentration were added dropwise into a 10% (w/v) sodium tripolyphosphate solution separately through a 25 gauge (25G) needle by using a syringe pump (GENIE, Kent Scientific Corp., Torrington, CT) at a pumping rate of 20ml/hr. The resulting chitosan microspheres were allowed to be cured in the polyphosphate solution for 24 hours. The microspheres were washed with distilled water and air dried for 48 hours. The dried microspheres were stored under vacuum in a dessicator.

Fabrication of 3-D chitosan matrices

Three dimensional chitosan matrices were developed from chitosan microspheres by a modified form of sintered microsphere fabrication technique previously developed in our laboratory [14]. Briefly, chitosan microspheres were mixed with a small amount of 3% (v/v) acetic acid and then transferred immediately into a stainless steel mold and heated in an 80°C

oven for 100 minutes. The mold was then allowed to cool down to room temperature. The resulting 3-D matrices were then removed and stored under vacuum in a desiccator for future use.

Morphology of chitosan microspheres and 3-D matrices

The morphology of chitosan microspheres and 3-D matrices were visualized using JEOL JSM-6400 scanning electron microscopy after coating with gold/palladium.

Mechanical evaluation of 3-D chitosan matrices

Compressive moduli of 3-D chitosan matrices (5 mm in diameter × 10 mm in length) were determined using an Instron 5544 mechanical tester at a crosshead speed of 5 mm/min.

MC3T3-E1 cell adhesion and proliferation on 3-D chitosan matrices

Three dimensional chitosan matrices (5 mm in diameter × 10 mm in length) were sterilized with 70% (v/v) ethanol and washed three times with sterile PBS before cell seeding. Matrices were placed into 24-well plates and seeded with MC3T3-E1 osteoblast-like cells (ATCC) cultured in Ham's F-12 medium supplemented with 10% FBS and 1% penicillin-streptomycin and were maintained at 37°C in an incubator with 5% CO_2 for 4, 7 and 14 days. The medium was changed every other day. At predetermined time, matrices with cells were washed with PBS to remove unattached cells. The cells on the scaffolds were fixed with 1% glutaraldehyde for 1 hour, 3% glutaraldehyde for another 24 hours followed by dehydration using alcohol series. Cell adhesion and proliferation on the matrices were visualized using SEM after coating the matrices with gold/palladium.

Statistical analysis

Statistical analysis was carried out using one-way analysis of variance (one-way ANOVA).

RESULTS AND DISCUSSION

Chitosan microsphere preparation, 3-D matrix fabrication and characterization

Figure 1(a) shows the SEM image of chitosan micropheres formed by ionotropic gelation. It has been found that the concentration of chitosan solution has a significant effect on the size of the microspheres formed. At low concentration (<1%) no microsphere formation was observed. Concentrations above 1.5% resulted in the formation of uniform solid microspheres. Table I shows the effect of the concentration of chitosan solution on the size of chitosan microspheres. It

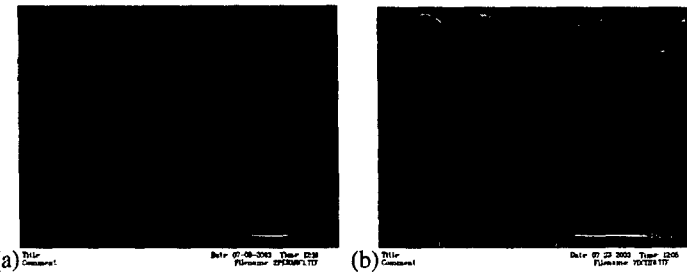

Figure 1 Scanning electron micrographs showing morphology of chitosan microspheres (a) and morphology of three dimensional matrices (b).

has been found that increasing the solution concentration resulted in significant increase in the microsphere size. Figure 1(b) shows the SEM of sintered three dimensional chitosan matrix showing the interconnected porous structure of the matrix. Table II shows the compressive moduli of the three dimensional chitosan matrices. It has been found that the 3-D matrices showed high mechanical moduli in the range of trabecular bone. No significant differences in the compressive modulus of the matrices were found by varying the size of the microspheres used for the fabrication of three dimensional matrices in the range studied.

***In vitro* cell adhesion and proliferation on chitosan matrices**

Figure 2(a) and (b) show the SEM images of MC3T3-E1 cells on the 3-D chitosan matrices after 4 days. Both well spread and slightly spread cells were present on the surface of 3-D matrices after 4 days in culture. Figure 2(c) and (d) show the SEM images of MC3T3-E1 cells on the 3-D chitosan matrices after 7 days. Mutilayer of well spread cells were present on the 3-D matrices after 7 days in culture. Figure 2(e) and (f) show the SEM images of MC3T3-E1 cells on the 3-D chitosan matrices after 14 days. SEM shows evidences of increased cell proliferation over the matrices and within the pore system. Cellular connections were evident at the bonding points between the microspheres.

Table I. Effect of chitosan solution concentration on the size of chitosan microspheres

Concentration (%)	1.5	2	2.5
Microsphere size[†] (μm)	668±25	795±36	888±26

[†] Significant difference between groups. ($p<0.05$)

Table II Effect of chitosan microsphere size on the compressive modulus of 3-D matrices

Microsphere size (μm)	668±25	795±36	888±26
Compressive modulus[‡] (MPa)	626.7±70.9	655.3±101.4	591.6±50.9

[‡] No significant difference between groups. ($p<0.05$)

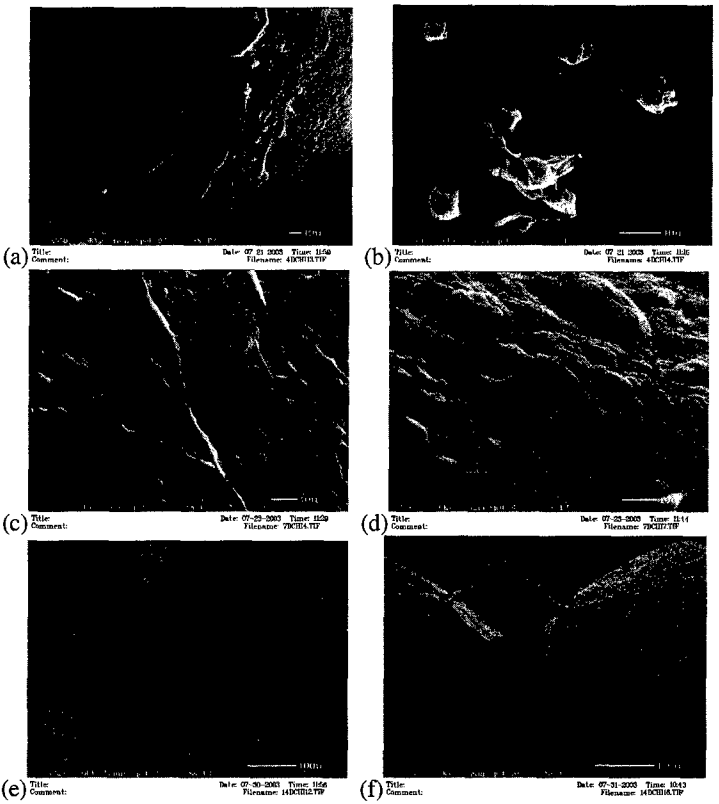

Figure 2. Scanning electron micrographs demonstrating osteoblast cells attachment and proliferation on chitosan matrices. (a) and (b): cell cultured for 4 days; (c) and (d): cell cultured for 7 days; (e) and (f): cell cultured for 14 days.

CONCLUSIONS

In this study, three dimensional chitosan matrices from chitosan microspheres having high compressive moduli in the range of trabecular bone were fabricated. The size of chitosan microspheres can be controlled by varying the concentration of chitosan solution. The three dimensional chitosan matrices were found to be highly osteoconductive making them potential candidates for bone tissue engineering applications.

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